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COMMUNICATIONS

Published on Web 10/21/2004

Two-State Folding Observed in Individual Protein Molecules

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The folding thermodynamics and kinetics of small proteins are often described in terms of a two-state model[1,2] in which only two populations of molecules exist (folded and unfolded) separated by a single free energy barrier. The simplicity of the corresponding time-dependent behavior should be reflected in the stochastic dynamics of individual proteins. Here we report single-molecule folding/unfolding trajectories of a protein that meets all criteria of the two-state model.[3,4] Förster resonance energy transfer (FRET) measurements were obtained from individual protein molecules trapped in surface-tethered lipid vesicles. The trajectories demonstrate the predicted bistable behavior with very fast steplike transitions between folded and unfolded conformations. The results provide the opportunity to examine the correspondence between single-molecule and ensemble measurements quantitatively. They stand in contrast to the unexpected complexity observed in previous reports of folding trajectories of individual proteins[5,6] and offer the first model-free demonstration of two-state protein-folding dynamics.

The cold-shock protein from *Thermotoga maritima* (Csp*Tm*) was labeled with a green-fluorescing and a red-fluorescing dye, serving as FRET donor and acceptor, respectively.[4] FRET upon excitation by a laser beam allows folded and unfolded molecules to be distinguished on the basis of the strong distance dependence of energy transfer between the chromophores. Previous experiments on single molecules freely diffusing in solution indicated that Csp*Tm* exhibits only two populations of molecules in its FRET efficiency distributions, corresponding to the folded and unfolded states.[4,7] To keep the labeled molecules in the laser beam long enough to observe multiple folding and unfolding events, they were individually encapsulated in unilamellar lipid vesicles, which were surface-tethered using biotin−avidin chemistry [5,8] (Figure 1). This method of immobilization was recently developed to allow long observation times for individual proteins while minimizing their surface interactions.[8] Encapsulation was carried out in aqueous buffer containing 2 M of the denaturant guanidinium chloride. Under these solution conditions, folded and unfolded states are equally populated.[4] A sample-scanning confocal microscope was used to locate individual molecules of Csp*Tm* and record fluorescence intensity traces. Donor and acceptor photons were collected separately, allowing the calculation of FRET efficiencies as a function of time. Single-molecule fluorescence depolarization experiments did not give any indication for interactions of trapped proteins with vesicular walls.

Two such measurements are shown in Figure 2, A and B. The data in these trajectories were binned in 20 ms intervals. Steady levels of FRET efficiency are followed by rapid jumps (seen in insets as anti-correlated changes of donor and acceptor intensities), until photobleaching of one of the dyes occurs. On the basis of previous FRET experiments with Csp*Tm*,[4] we identified high transfer efficiencies with the folded state and low transfer efficiencies with the unfolded state of the protein. Consequently, abrupt changes in the FRET efficiency (with an average amplitude of 0.46 ± 0.16) represent folding or unfolding events. The actual transitions were too rapid to be time-resolved and occurred within the time span of one or two time bins, even when the data were analyzed with a much smaller integration time of 100 μs. One example for such an analysis is shown in Figure 2C. Due to the high FRET efficiency of the folded protein, the donor essentially stops emitting after the transition (green arrow). It can then be estimated that the transition occurs within 100−200 μs of the last registered donor photon. A statistical analysis was performed in which the likelihood to find a specific experimental realization of donor and acceptor photon sequences was evaluated, taking into account the photon emission probabilities before and after the transition. This analysis confirmed the visual estimate.

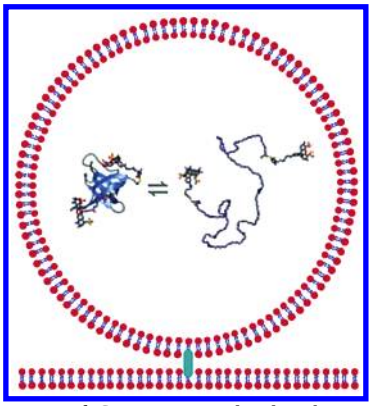

Figure 1. Schematic of Csp*Tm* encapsulated within a surface-tethered vesicle (relative dimensions not to scale).

To quantitatively compare our results to ensemble experiments, we built a histogram of all time intervals preceding folding and unfolding transitions in the trajectories of 43 individual protein molecules (54 transitions overall, Figure 3). An exponential fit to this histogram gave a rate constant of 0.62 ± 0.26 s^{-1} (95% confidence bounds). This rate is in agreement with the folding rate constant of 0.39 ± 0.02 s^{-1} measured in a bulk experiment under identical solution conditions.[4]

It is a characteristic of two-state systems that, upon perturbation of an ensemble of molecules, the distribution between the two states relaxes with an exponential time course, the rate of which is determined by the height of the barrier. The single-molecule equivalent of this behavior is stochastic but fast jumping between the two states.[9] In the latter case, the observable determined by the barrier height is the frequency of transitions. The steplike folding and unfolding behavior seen in our experiment is exactly what would be predicted from this notion and is in quantitative agreement with the ensemble result. The second important parameter, apart from the transition frequency, is the actual time the molecule spends crossing the barrier. Our results provide an upper bound of ∼200 μs for this transit time, which is in agreement with the simple assumptions made in previous analyses of protein folding.[10] In a Kramers-type formulation of escape over a barrier,[11] the barrier-

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10.1021/ja046209k CCC: $27.50

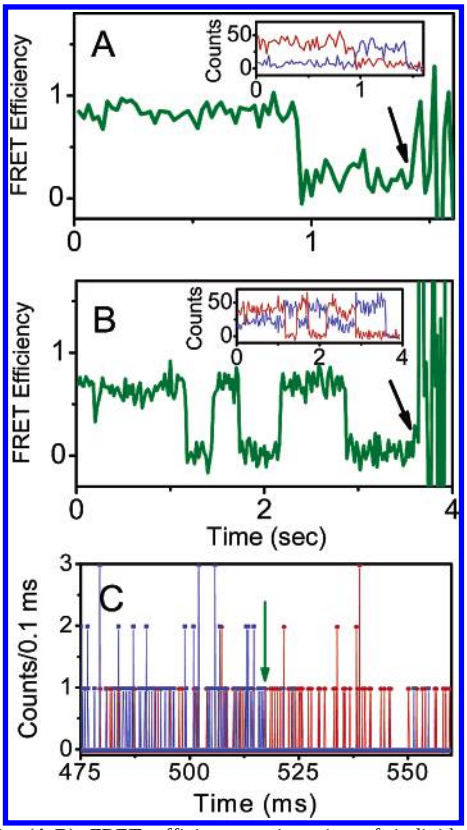

Figure 2. (A,B) FRET efficiency trajectories of individual Csp*Tm* molecules trapped in vesicles at 2 M GuHCl showing (A) a single or (B) multiple abrupt folding/unfolding transitions with a binning time of 20 ms. Arrows indicate the time of photobleaching of one of the dyes. (Inset) Fluorescence intensity trajectories from which FRET efficiencies were calculated. (Blue) Donor signal. (Red) Acceptor signal. (C) A single-molecule trajectory analyzed with 100-μs bins. Green arrow indicates time of folding transition.

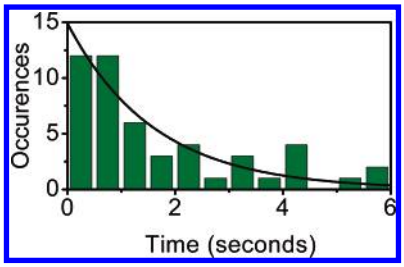

Figure 3. Histogram of time intervals preceding transitions in Csp*Tm* single-molecule trajectories, fit to a single-exponential decay (black). The rate constant obtained from the fit, 0.62 ± 0.26 s^{-1} (95% confidence bounds), agrees well with the ensemble-averaged folding rate constant.

crossing time is a function not only of the effective diffusion coefficient of the folding molecule as it traverses the barrier but also of the shape of the barrier. The direct measurement of transition times in single-molecule experiments of the type described here will therefore provide essential constraints on these fundamental parameters of the free energy barrier that are difficult to obtain from ensemble-averaged experiments.[12−14]

In previous work on single-molecule protein folding, correlation analysis [15] and histograms[4,7,15,16] constructed from measurements on freely diffusing or immobilized proteins were used to demonstrate the presence of the two populations of molecules expected from ensemble experiments. However, none of these approaches allowed the direct observation of folding transitions in individual molecules. Additionally, it was shown that single-molecule histograms can hide more complex behavior.[5] The prototypical two-state behavior observed here in Csp*Tm* trajectories is in contrast with recent reports of folding trajectories of individual proteins. Rhoades et al., using single-molecule FRET spectroscopy, showed that some of the folding/unfolding transitions seen in a large protein, adenylate kinase, were very slow and took up to a few seconds to complete.[5] Fernandez and Li investigated covalent heptamers of ubiquitin molecules using force-clamp microscopy and found slow, continuous folding trajectories.[6] However, in monomeric two-state proteins such as Csp*Tm*, the involvement of non-native interactions is minimal, and the resulting robustness of their folding behavior may contribute to eliminating the additional complexity observed in previous experiments.

Acknowledgment. We would like to thank William Eaton, Attila Szabo, Gerhard Hummer, and Eli Pollak for discussions, as well as Moshe Pollak and Guy Ziv for help with statistical analysis. This work was partially funded by grants from the Israel Science Foundation and the U.S.-Israel Binational Science Foundation (to G.H.), as well as the Deutsche Forschungsgemeinschaft (to B.S.).

Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA046209K

Rhoades et al., Communication to JACS
Supporting Information

Additional Experimental Details

Cysteine residues were introduced at the chain termini of Csp*Tm* by site-directed mutagenesis to provide functional groups for the specific attachment of the dyes. Expression and purification were performed as described previously [1]. Dye labeling was carried out by procedures described by the manufacturer, Molecular Probes. Alexa Fluor 488 maleimide was reacted with the protein, and singly-labeled protein separated from unlabeled and doubly-labeled protein by ion exchange chromatography (Mono Q HR 5/5, Amersham Pharmacia). The fractions containing singly-labeled Csp*Tm*, as confirmed by electrospray ionization mass spectroscopy, were labeled with Alexa Fluor 594 maleimide. Doubly labeled protein was again separated from singly labeled protein by ion exchange chromatography.

Vesicles were prepared as described before [2]. Csp*Tm* molecules at a concentration of 3 μM were included in the hydration buffer, ensuring less than one molecule per vesicle on the average. The vesicles were separated from unencapsulated protein on a Sepharose 2B gel filtration column. They were then tethered to a lipid membrane supported on a glass cover slip, using biotin-avidin chemistry [2]. All measurements were done using a home-built sample-scanning confocal microscope [2], operated in the time-stamping mode in which the arrival time of each photon at one of the detectors is registered . Individual Csp*Tm* molecules trapped in lipid vesicles were excited with a 488 nm laser beam. The laser intensity was kept low (1 μW) in order to delay photodestruction of the fluorophores. A dichroic mirror (565DCLP, Chroma) was used to separate the fluorescence signals emitted by the donor and acceptor. Bandpass filters (D535/40 for donor and D620/60 for acceptor, both from Chroma) positioned in front of the detectors ensured minimal cross-talk between the two channels.

Energy transfer efficiency values were calculated according to $E=I_A/(I_A+I_D)$, where I_A and I_D are the acceptor and donor intensities, respectively. This expression does not take into account possible differences in detection efficiencies and fluorescence quantum efficiencies between donor and acceptor, the inclusion of which does not affect the results of the paper. More than 500 single molecule trajectories were analyzed, out of which 43 molecules showed one or more transitions between folded and unfolded states. Identification of transitions strictly relied on anti-correlated changes in donor and acceptor channels. Data was typically binned with 20 ms bins, but we also used 100 μs bins in an attempt to resolve transitions. The location of a transition was estimated both visually and using a statistical analysis of the data. To perform the latter, we used Bayes theorem to relate the probability to find a transition at a certain time T to the probability to find a specific experimental realization of donor and acceptor photon sequences, given a transition at T. The latter probability could be calculated directly from the experimental data, taking into account the measured arrival times of all photons at the detectors, as well as the FRET efficiencies and emission rates before and after the transition, which were calculated from time-averaged trajectories. All error values stated in the paper are given as standard deviations unless otherwise noted.

In order to test the possibility of interaction between encapsulated molecules and vesicular walls we conducted control experiments using single-molecule fluorescence polarization spectroscopy [3]. Molecules were excited with circularly-polarized light and their emission was split by a cube polarizer into vertical and horizontal components, detected with two avalanche photodiodes, and the fluorescence polarization was calculated. Polarization changes, which might have indicated interaction of the molecules with vesicular walls, were not observed. In addition, a very narrow distribution of the polarization values from a series of single-molecule measurements was also indicative of unhindered rotational motion of the molecules within the vesicles [2]. Future two-color excitation experiments monitoring fluorescence intensities and polarization simultaneously will provide the means to control for photochemical effects and the possible effect of surface interactions during each trajectory.

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